Mammalian Biology 81 (2016) 163–175

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.com/locate/mambio

Original Investigation

Mitogenomics of the mountain tapir (*Tapirus pinchaque*, Tapiridae, Perissodactyla, Mammalia) in Colombia and Ecuador: Phylogeography and insights into the origin and systematics of the South American tapirs

Manuel Ruiz-García[a,*], Armando Castellanos[b], Luz Agueda Bernal[a], Myreya Pinedo-Castro[a], Franz Kaston[c], Joseph M. Shostell[d]

[a] *Laboratorio de Genética de Poblaciones-Biología Evolutiva, Unidad de Genética, Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Cra 7A No 43-82, Bogotá, DC, Colombia*
[b] *Andean Bear Fundation, La Isla, Quito, Ecuador*
[c] *Fundación Nativa, Apartado Aéreo 59199, Bogotá, DC, Colombia*
[d] *Department of Math Science and Technology, University of Minnesota Crookston, Crookston, MN 56716, USA*

ARTICLE INFO

Article history:
Received 16 June 2015
Accepted 11 November 2015
Handled by Frank E. Zachos
Available online 19 November 2015

Keywords:
Tapirus pinchaque
Mitochondrial DNA
Genetic diversity
Spatial genetic structure
Phylogenetic analyses

ABSTRACT

We sampled 45 Andean mountain tapirs (*Tapirus pinchaque*) from Colombia and Ecuador and sequenced 15 mitochondrial genes (two rRNA and 13 protein codifying genes)—making up 13,939 base pairs, approximately 83.1% of the total mitochondrial DNA's length. The overall sample had low to medium levels of nucleotide diversity with diversity slightly higher for the Colombian population. Both populations experienced high historical gene flow and our genetic heterogeneity analyses revealed a low genetic differentiation between them. Therefore, we did not detect any molecular subspecies, or significantly different evolutionary units for *T. pinchaque*. This species experienced a population expansion in the last 100,000 years but this expansion was more pronounced in the Ecuadorian population especially in the last 10,000 years, whereas the Colombian population underwent a strong bottleneck in the last 5,000 years. There was no significant spatial trend in genetic structure for the mountain tapir in Colombia and Ecuador. Phylogenetic analyses did not detect any important geographic clade within this species. Temporal split between *T. pinchaque* and *T. terrestris* might have occurred around 7–1.5 million years ago (MYA). *T. pinchaque* and *T. terrestris* + *T. kabomani* are two monophyletic clades, suggesting that *T. kabomani* is not a full species.

Introduction

An important first step in protecting groups of similarly structured organisms is to place them into discrete taxa. Once a species is recognized we monitor its overall fitness and develop conservation plans towards its benefit. Unfortunately, biologists do not always agree on the naming of new species in part due to the species concept they select (i.e. Phylogenetic Species Concept, PSC, vs. Biological Species Concept, BSC) as well as the data (fossils, DNA results, morphology, ethology, ecology) they use. One example of this non-agreement is in the genus *Tapirus*. In Latin America three tapir species have been traditionally accepted, the Baird or Central American tapir (*Tapirus bairdii*), the mountain tapir (*T. pinchaque*) and the lowland or Brazilian tapir (*T. terrestris*). There is also the Malayan tapir species (*T. indicus*) in Asia (Brooks et al., 1997).

T. pinchaque (Roulin 1829) is the smallest of the four living tapir species—having an average shoulder height of 0.90 m, body length of 1.8 m and weight of 150 kg. This species is found in habitats of 1,700–4,800 m above sea level (masl) (Downer, 2001; Schauenberg, 1969) in different types of north Andean forests and páramos (open grass lands) in Colombia, Ecuador, and in Northern Peru. It is an extremely efficient seed disperser. Downer (2001) documented more than 50 Andean plant species that have germinated in tapir feces within the Sangay National Park (Ecuador). Mountain tapir survival is considered a crucial factor for the conservation of the northern Andean wilderness and watershed, an area containing the most important water reserves in this and surrounding areas.

* Corresponding author. Tel.: +57 1 3208320x4085; fax: +57 1 3208320x4085.
E-mail addresses: mruizgar@yahoo.es, mruiz@javeriana.edu.co (M. Ruiz-García).

http://dx.doi.org/10.1016/j.mambio.2015.11.001
1616-5047/

This tapir is currently classified as an endangered species in the IUCN Red List (IUCN 2011) at the global level and it is also enclosed in Appendix I in CITES, being one of the rarest large mammals of the world. Moreover, they have been categorized as critically endangered in the Andean area of Colombia, Ecuador and Peru (Lizcano et al., 2006; Tirira, 2011).

The origin of tapirs in South America and the role played by *T. pinchaque* in this origin are very controversial topics. Hershkovitz (1954) analyzed morphological characters and concluded that the ancestor of *T. pinchaque* was the first tapir form to enter South America prior to the formation of the Panamanian Isthmus more than 3–4 MYA. Later, the ancestors of *T. terrestris*, *T. bairdii* and other tapirs from hotter climates migrated into South America. Therefore, the ancestors of the three tapir species currently present in South America previously appeared in North America at a time when the Isthmus of Panama was not yet formed. Three independent lineages evolved and, according to Hershkovitz (1966), *T. bairdii* is the most recent. This author distinguished two subspecies of *T. pinchaque*: *T. p. pinchaque* (Roulin; type locality in Páramo de Sumapaz in Cundinamarca, Colombia) and *T. p. leucogenys* (Gray; type locality in Páramo de Azuay in the Eastern Cordillera of Ecuador).

Haffer (1970) introduced a different hypothesis on the origin of the tapirs of South America taking into account morphological and biogeographical considerations. He considered the ancestor of *T. pinchaque* to be the first form which colonized the Northern Andes during its last rising within the Pliocene. During the Pleistocene, the ancestor of *T. pinchaque* gave rise in the eastern lowlands to the ancestor of *T. terrestris* and in the western lowlands (Choco refugium) to the ancestor of *T. bairdii*, which later migrated to Central America (one unique lineage hypothesis). An alternative hypothesis suggests that the ancestor of *T. bairdii* originated in a Central American refugium and then migrated towards the Pacific area of what would eventually become the western section of current Colombia (Haffer 1970).

Ashley et al. (1996) used mitochondrial *COII* sequences to determine that the ancestor of *T. bairdii* diverged from the ancestor of the two strictly South American tapir species around 19–20 MYA. The temporal split between the ancestors of *T. terrestris* and *T. pinchaque* was estimated around 3 MYA. Norman and Ashley (2000) estimated new temporal splits. For the ancestors of *T. bairdii* and *T. terrestris* + *T. pinchaque*, they ranged from 18 to 20.4 MYA (*COII*), or from 15 to 16.5 MYA (*12S rRNA*). These genes supported divergence times between the ancestors of *T. terrestris* and *T. pinchaque* of 2.5–2.7 MYA and 1.5–1.6 MYA, respectively. Ruiz-García et al. (2012) used mitochondrial *Cyt-b* sequences and a Bayesian tree to determine that the ancestors of *T. terrestris* and *T. pinchaque* diverged around 3.8 MYA. Using a Median Joining Network, the most frequent *T. terrestris* haplotypes diverged from the main *T. pinchaque*'s haplotype around 1.55 ± 0.32 MYA.

Recently, Cozzuol et al. (2013) claimed the existence of a new tapir species. They compared *Cyt-b* gene sequences of four Amazon tapir individuals (two from Brazil and two from Colombia) with 45 *Cyt-b* gene sequenced from Thoisy et al. (2010). Additionally, they included results for two mitochondrial genes (*COI* and *COII*) for six *T. terrestris*, one *T. pinchaque* and three individuals of *T. kabomani*. They concluded that the three mitochondrial genes showed the existence of *T. kabomani* as a full species. Voss et al. (2014) however, questioned the validity of *T. kabomani* as a different species from *T. terrestris*.

We sequenced the mitochondrial genomes of 45 *T. pinchaque* individuals sampled throughout six populations of Colombia and Ecuador. These two countries harbour more than 98% of the living mountain tapirs.

The main aims of the present work are as follows: (1) To determine mitochondrial diversity levels in *T. pinchaque*; (2) To determine levels of genetic differentiation between *T. pinchaque* populations to provide new results testing for the possible existence of subspecies within mountain tapirs (sensu Hershkovitz, 1954); (3) To analyze possible historical demographic changes throughout the evolution of *T. pinchaque*; (4) To determine spatial genetic structure in *T. pinchaque*; and (5) To contribute with new data to our understanding of speciation in the *Tapirus* genus.

Material and methods

Out of 80 *T. pinchaque* samples from Colombia and Ecuador we selected 45 (high-quality DNA) for mitochondrial DNA sequencing. Additionally, 17 samples of *T. terrestris*, 13 samples of *T. bairdii* and 7 samples of *T. indicus* were also sequenced. The origins of samples are in Table 1 and Fig. 1. *T. pinchaque* samples were obtained from hunted and captive animals as well as from tapirs captured for research (radio tracking). Some were from protected areas in Colombia and Ecuador (Table 2).

DNA of high quality was extracted and isolated from blood and muscle samples using the QIAamp DNA Mini Kit (Qiagen, Inc.). Amplifications were carried out using the LongRange PCR Kit (Qiagen, Inc.), with a final volume reaction of 25 μl. The reaction mix was composed of a 80–200 ng DNA template, 2 units of Long-Range PCR Enzyme, 3 μl of 10× LongRange PCR Buffer, 4 μl (15 pmol) of each primer, and 2 μl of 10 mM dNTPs. The cycling conditions were 95 °C for 3 min, followed by 50 cycles denaturing at 95 °C for 20 s, primer annealing at 53–58 °C (with a decrease of 0.1 °C every cycle) for 30 s, and extension at 72 °C for 10 min. This was followed by 30 cycles of denaturing at 95 °C for 20 s, annealing at 48–53 °C for 30 s, and extension at 72 °C for 5 min, with a final extension at 72 °C for 10 min. All amplifications, including positive and negative controls, were checked onto 2% agarose gels under a Hoefer UV Transilluminator. Both mtDNA strands were sequenced directly using BigDye Terminator v3.1 (Applied Biosystems, Inc.). We used a 377A (ABI) automated DNA sequencer.

We used four sets of primers (Table 3) to generate overlapping amplicons from 3,345 bp to 5,049 bp in length. These amplicons allowed us to carry out a quality test for genome circularity (Bensasson et al., 2001; Thalmann et al., 2004). Herein, we show the results of 15 mitochondrial genes (two rRNA and 13 protein codifying genes; Table 4). The sequences were concatenated by means of the SequenceMatrix v. 1.7.6 (Vaidya et al., 2011). They covered 13,939 bp which represented about 83.1% of the total mitochondrial DNA length. Overlapping regions were examined for irregularities, such as frameshift mutations and premature stop codons. All the population genetics analyses carried out were using the sequences of these 15 mitochondrial genes.

Additionally, we analyzed the phylogenetic relationships between *T. pinchaque* and *T. kabomani* using three concatenated mitochondrial genes (*Cyt-b* + *COI* + *COII*), the same genes used by Cozzuol et al. (2013). They published a tree including one sample of *T. pinchaque*, six of *T. terrestris* and three of *T. kabomani*. As *T. kabomani* was not included in the clade of *T. terrestris* + *T. pinchaque*, they concluded *T. kabomani* to be a new full species. To investigate this question, we sequenced 93 Neotropical tapir specimens (46 *T. pinchaque*, 29 *T. terrestris*, 5 *T. kabomani* – including the two Brazilian specimens of Cozzuol et al., 2013, and 13 *T. bairdii*) for *Cyt-b*, *COI* and *COII*. The origins of the five *T. kabomani* are in Table 1.

Population genetics analyses

The Modeltest 3.7 (Posada and Crandall, 1998) and the Mega 5.1 Softwares (Tamura et al., 2011) were applied to determine the best evolutionary nucleotide model for sequences of *T. pinchaque*, using the Akaike Information Criteria (AIC; Akaike, 1974) to determine the best evolutionary nucleotide substitution model.

Table 1
Countries, number of individuals, exact geographic origins and the mitogenomes determined for the 45 *Tapirus pinchaque*, 17 *Tapirus terrestris*, eight *Tapirus bairdii* and seven *Tapirus indicus* individuals sequenced at 15 mitochondrial genes.

Country	Geographic origins	Number of animals	Mitogenomes found in each locality
Tapirus pinchaque			
Colombia	Los Nevados National Park in the Caldas Department	3	M1, M2
	Los Nevados National Park in the Otún area in Risaralda Department	3	M1, M2
	Los Nevados National Park in the El Bosque area in Risaralda Department	2	M2, M4
	Gaitania in Tolima Department	3	M1, M2,M15
	La Planada (La Bella and San Miguel), Marquetalia in Tolima Department	3	M1, M19, M21
	Hereje River in Tolima Department	1	M15
	Saldaña River in Tolima Department	1	M16
	La Azulena in Tolima Department	1	M1
	Peñas Blancas in Tolima Department	1	M1
	Nevado del Huila National Park	1	M20
	Puracé National Park in Huila Department	1	M3
Ecuador	Cuyuja, Quijos, Napo Province	6	M1, M6, M7, M12, M13, M14
	Oyacachi, Chaco, Napo Province	3	M1, M10, M11
	Alto Papallacta River, Cayambe-Coca National Park, Napo Province	9	M1, M5, M6, M7, M8, M9
	Cosangua, Napo Province	2	M13, M18
	La Bonita, Sucumbios Province	1	M3
	Sangay National Park (Las Culebrillas), Morona-Santiago Province	3	M12, M14,M17
	Podocarpus National Park, Loja Province	1	M13
Tapirus terrestris			
Colombia	Yali, Caqueta	1	M22
	Puerto Berrio, Antioquia	1	M23
	Remedios, Antioquia	1	M24
	Sabanacules, Antioquia	1	M25
Ecuador	Reserva Natural Cuyabeno	1	M26
Peru	Amaruyama, Madre de Dios River	1	M27
	Reserva Ecologica Tariyaca, Madre de Dios River	1	M28
	Parque Nacional Bahuaja-Sone	1	M29
	Tournavista, Pachitea River	1	M30
Bolivia	Ibare River	1	M31
	San Jose de Chiquitos	1	M32
	Trampa del Tigre	1	M33
Argentina	Chaco	1	M34
Brazil	Javari River	1	M35
Suriname	Magali	3	M36
Tapirus bairdii			
Colombia	Choco	1	M37
Panama	Darien	8	M37, M38, M39, M40, M41, M42
Costa Rica	Parque Nacional Braulio Carrillo	3	M43
Mexico	Yucatan	1	M44
Tapirus indicus			
Malaysia		3	M45, M46
Thailand		3	M46, M47
Sumatra		1	M48
Tapirus kabomani			
Brazil	Southern Amazon Department	1	Cozzuol et al. (2013)
	Rondonia Department	1	Cozzuol et al. (2013)
Colombia	San Martin de Amacayacu, Amazon River	1	This work
Peru	Mazan River, affluent Napo River, Loreto, Northern Peruvian Amazon	1	This work
Ecuador	Tena, Upper Napo River, Ecuadorian Amazon	1	This work

The following statistics to determine the genetic diversity for the overall sample of *T. pinchaque* and for the samples separately from Colombia and Ecuador were employed: number of haplotypes (H), haplotype diversity (H_d), nucleotide diversity (π), average number of nucleotide differences (k) and θ statistic by sequence. These genetic diversity statistics were calculated with the Programs DNAsp 5.1 (Librado and Rozas, 2009) and Arlequin 3.5.1.2 (Excoffier and Lischer, 2010).

We used different tests to measure genetic heterogeneity and possible indirect gene flow estimates among the *T. pinchaque* populations. Six geographical populations were considered, three in Colombia and three in Ecuador because there were six discrete geographical areas separated by considerable geographical distances. We estimated the G_{ST}, γ_{ST}, N_{ST} and F_{ST} statistics (Hudson et al., 1992). Significance was estimated with permutation tests using 10,000 replicates.

We carried out an AMOVA analysis of the *T. pinchaque* sample to determine the distribution of genetic diversity at different hierarchical geographical levels (Excoffier et al., 1992). Two groups were considered (Colombia and Ecuador) and within these groups, six populations (three in Colombia: Caldas-Risaralda, Tolima and Huila; three in Ecuador: Napo, Sangay and Podocarpus). The fixation indices of Wright (1951) were estimated in the AMOVA analysis: Φ_{sc} (variation of populations within the groups), Φ_{ct}

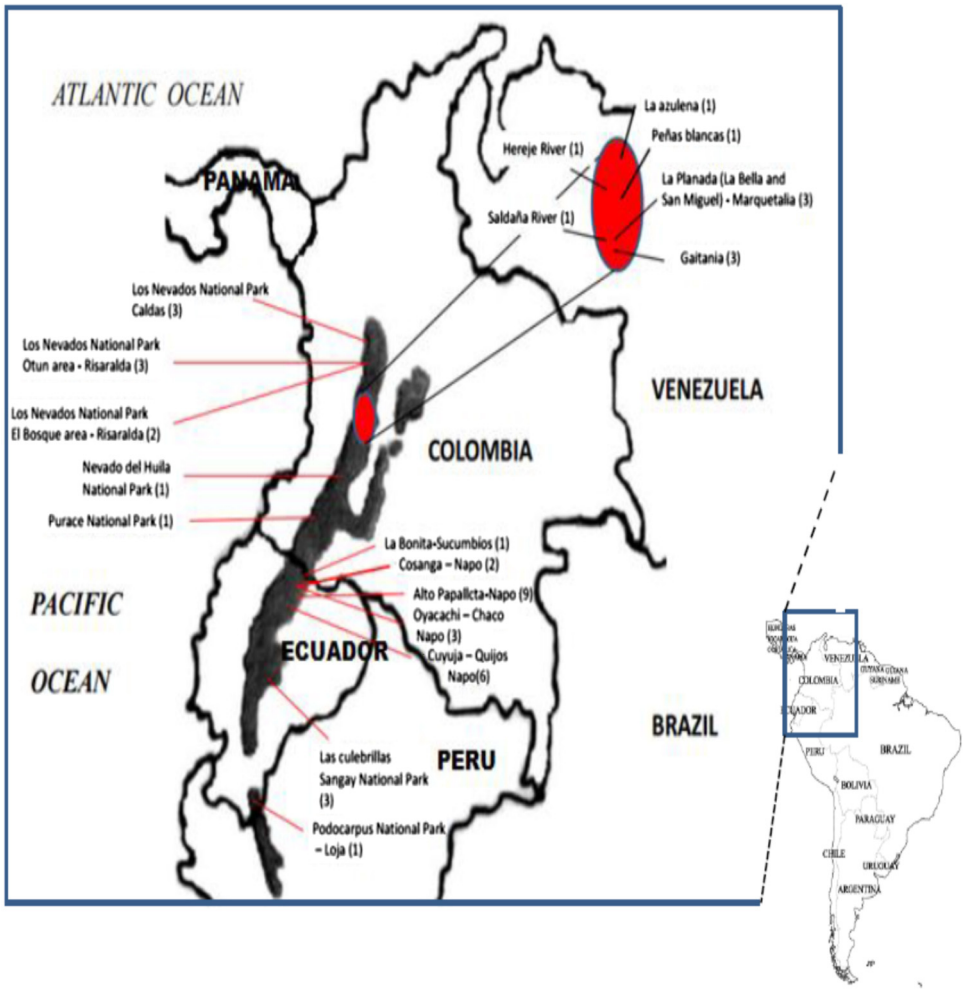

Fig. 1. Map with the areas where 45 *Tapirus pinchaque* individuals were sampled in Colombia and Ecuador.

(variation among groups) and Φ_{st} (variation among populations). Analyses were carried out using Arlequin 3.5.1.2 Software.

A Bayesian skyline plot (BSP) was obtained by means of the BEAST v. 1.6.2 (Drummond and Rambaut, 2007) and Tracer v1.5 Softwares to determine possible demographic changes across the natural history of *T. pinchaque*. The Coalescent-Bayesian skyline option in the tree priors was selected with three steps and a piecewise-constant skyline model with 60,000,000 generations. Marginal densities of temporal splits were analyzed and the Bayesian Skyline reconstruction option was selected for the tree log files. A stepwise (constant) Bayesian skyline variant was selected with the maximum time as the upper 95% high posterior

Table 2
Protected areas (in km^2) in Colombia, Ecuador and Peru with confirmed presence of *Tapirus pinchaque*; estimations of low and high number of mountain tapirs in these areas, following Cavelier et al. (2010) and which of these protected areas were represented in our mitogenomic analysis. NP = National Park.

Country	Place	Area (km^2)	Lower estimation	Upper estimation	Analyzed in this study
Colombia	Los Nevados NP	98,503	17	18	Yes
	Las Hermosas NP	301,045	51	55	No, but some samples were from very nearby areas
	Nevado del Huila NP	1,084,443	185	197	Yes
	Purace NP	618,197	105	112	Yes
	Cueva de Los Guacharos NP	39,816	7	7	No
	Alto Fragua-Indi Wasi	64,463	11	12	No
	Sumapaz NP	714,590	122	130	No
	Cordillera de los Picachos NP	264,495	45	48	No
Ecuador	Cayambe-Coca NP	1,408,606	245	261	Yes
	Sumaco Napo Galeras NP	525,865	90	95	No
	Antisana NP	617,042	105	112	No
	Llanganatis NP	824,778	141	150	No
	Sangay NP	1,294,180	220	235	Yes
	Podocarpus NP	1,069,749	182	194	Yes
Peru	Tabaconas-Namballe	238,907	41	43	No
Total		9,194,679	1,567	1,669	

Table 3
Long range amplification primers employed in this work.

Primer name	Primer sequence (5′ to 3′)	Amplicon sizes (bp)
TPF1	5′-CTAACTCCATAAAACA-3′	3,768
TPR1	5′-CAAAATCCTCCGT-3′	
TPF2	5′-AAACATAAGAAAT-3′	4,990
TPR2	5′-ATCAGCCCTCCTTA-3′	
TPF3	5′-TAGCCTTAATTCAA-3′	5,049
TPR3	5′-TAATTTCCCCATTA-3′	
TPF4	5′-TCAAACCAAAAAGG-3′	3,345
TPR4	5′-CAGGTTTGATCCT-3′	

density (HPD). The time during which the demographic changes were recorded was chosen by the software with a mutation rate of 2.5% per million of year (Ruiz-García et al., 2015).

We used IBD Software (version 1.2) (Bohonak, 2002) to determine possible correlation between the genetic distances (Kimura 2P distance; Kimura, 1980) and the geographical distances among these populations. A Mantel's test (Mantel, 1967) was used to determine the significance between the genetic distance and geographic distance matrices. The intercept and the slope of this relationship were calculated using Reduced Major Axis (RMA) regression (Sokal and Rohlf, 1981). Ten thousand randomizations were carried out to determine 99% confidence intervals. The calculations were completed with non-transformed and ln transformed data (genetic distance & geographical distance).

Phylogenetic analyses

Two phylogenetic trees were obtained for the complete 15 mitochondrial genes concatenated (13,939 bp). The first one was calculated with the maximum likelihood procedure (ML; Felsenstein, 1981) with the General Time Reversible model (GTR + G + I; G = gamma distributed rate variation among sites; I = constant proportion of invariable sites), being the best nucleotide substitution model in our data found by the Modeltest 3.7 Software (Ln = −25,474.80). This tree was obtained with the PAUP*4.0b8 Program (Swofford, 2002). The second one was a Bayesian tree (BI; Mau, 1996; Mau et al., 1999; Rannala and Yang, 1996). It was also based on the GTR + G + I model. This Bayesian analysis was carried out with the BEAST (v1.6.2) Software (Drummond and Rambaut, 2007).

Two runs of analyses were done, assuming a Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution (Drummond et al., 2006). Results from the two independent runs (60,000,000 generations with the first 6,000,000 discarded as burn in and parameter values sampled every 10,000 generations) were combined with LogCombiner Software (v1.6.2) (Drummond and Rambaut, 2007). The final tree was estimated using TreeAnnotator Software (v1.6.2) (Drummond and Rambaut, 2007) and visualized in the FigTree v1.3.1 Program. Additionally, the BEAST (v1.6.2) Software was run to estimate the time to the most recent common ancestor (TMRCA) for the different haplotype lineages found in different tapir species. As priors, we took a temporal split of 15 ± 2 MYA between the ancestor of *T. indicus* and those of the Latin American tapirs. We also used the temporal split of 7.5 ± 1 MYA between the ancestor of *T. bairdii* and those of the South American tapir species following Cozzuol et al. (2013). These estimates were recorded from the fossil record (Ferrero and Noriega 2007; Holanda et al., 2011; Holanda and Ferrero, 2013).

Additionally, we created a maximum likelihood tree, for the 93 tapirs sequenced at the concatenated *Cyt-b* + *COI* + *COII* genes to analyze the relationships among *T. pinchaque*, *T. terrestris* and *T. kabomani*.

Table 4
Fifteen mitochondrial genes sequenced and sizes in base pairs (bp) for 45 *Tapirus pinchaque*, 17 *Tapirus terrestris*, eight *Tapirus bairdii* and seven *Tapirus indicus*.

Mitochondrial genes sequenced	Sizes (bp)
12S rRNA	949
16S rRNA	1,578
ND1	956
ND2	1,041
ND3	345
ND4L	296
ND4	1,377
ND5	1,820
ND6	526
Cyt-b	1,139
COI	1,544
COII	683
COIII	783
ATP8	203
ATP6	680

Results

Genetic diversity

The maximum likelihood estimate of Transition/Transversion was 2.37 (Ln = −26,308.12). For a GTR model, the maximum likelihood estimate of the gamma parameter for sites rates was 0.2037 (+ G; five categories) (Ln = −25,481.66).

The genetic diversity level for the global *T. pinchaque* sample analyzed for the 15 mitochondrial genes concatenated showed 21 different haplotypes with $H_d = 0.904 \pm 0.003$ (high genetic diversity) and $\pi = 0.0041 \pm 0.0003$ (low-medium genetic diversity) (see Table 5). The genetic diversity levels for the Colombian and the Ecuadorian mountain tapir populations were similar, although the most unbiased gene diversity statistics were somewhat higher for the Colombian population.

Genetic heterogeneity tests (Table 6A) revealed a low genetic differentiation level ($G_{ST} = 0.0231$, $\gamma_{ST} = 0.0534$, $N_{ST} = 0.0558$ and $F_{ST} = 0.056$; P = 0.714–0.367, no significant), with the gene flow estimates showing that the populations were not disconnected and/or their separation was very recent (Nm = 1.58–9.15). F_{ST} analysis (Table 6B) showed that only three population comparisons out of 15 were significant. A gene flow comparison of population pairs only revealed 4 out of 15 cases with values lower than one.

The AMOVA revealed that the percentage of variation between groups (Colombia and Ecuador) was very small (2.27%) and not significant ($\phi_{CT} = 0.0227$, $P = 0.1026 \pm 0.0029$). The percentage of variation among the populations within the groups was also small (6.76%) and not significant ($\phi_{SC} = 0.0692$, $P = 0.1507 \pm 0.0038$). The major percentage of genetic variance within populations was 90.97%, but the difference between all the populations was not significant ($\phi_{ST} = 0.0903$, $P = 0.1381 \pm 0.0033$).

Historical demographic changes and spatial structure

The BSP analyses (Fig. 2) supported a continuous population expansion for the total population throughout the last 100,000 years, with the highest increase of females in the last 25,000 years. The Ecuadorian sample also yielded a continuous increase in female numbers within the last 25,000 years but this increase was more noteworthy in the last 10,000 years. The Colombian sample presented a more constant population size in the last 100,000 years, with a strong decrease in the last 5,000 years.

The four regression models did not show any significant relationship between genetic and geographic distances. The Mantel test between the ln(genetic distance) and the ln(geographic distance),

Table 5
Genetic diversity statistics for *Tapirus pinchaque* for the overall sample, for the Colombian sample and for the Ecuadorian sample at the 15 mitochondrial genes sequenced. Number of haplotypes (NH), haplotype diversity (H_d), nucleotide diversity (π), average number of nucleotide differences (K) and θ statistic ($=2N_e\mu$; N_e = effective female population size; μ = mutation rate per generation) by sequence.

	NH	H_d	π	K	θ per sequence
Total sample studied of *Tapirus pinchaque*	21	0.904 ± 0.003	0.0041 ± 0.0003	4.457 ± 0.238	15.094 ± 0.582
Colombian sample studied of *Tapirus pinchaque*	9	0.816 ± 0.013	0.0053 ± 0.0009	5.726 ± 0.862	13.248 ± 1.799
Ecuadorian sample studied of *Tapirus pinchaque*	14	0.937 ± 0.013	0.0029 ± 0.0007	3.200 ± 0.710	6.356 ± 1.366

for instance, showed r = −0.127 (p < 0.691 from 10,000 randomizations).

Phylogenetics of T. pinchaque

The ML tree (Fig. 3A) did not yield comprehensive clades reflecting geographical distribution, which agreed quite well with the IBD analysis. There were only some small clades with animals of related geographical areas. These clades comprised five Colombian animals from Los Nevados National Park and Tolima (bootstrap 85%) and tree animals from the Ecuadorian Napo Province (49%). One individual from La Planada (Tolima, Colombia) was the most differentiated of all the *T. pinchaque* studied. The BI tree used *T. indicus* as outgroup and it showed the first split by the ancestor of *T. bairdii* (p = 1) and later the ancestors of *T. terrestris* and *T. pinchaque* (p = 1) (Fig. 3B). The two most divergent *T. pinchaque* were two individuals from La Planada-Tolima (Colombia) and Chaco-Oyacachi-Napo (Ecuador) (p = 0.9 and 1, respectively). The well-supported clades were the same as those revealed in the previous tree. Therefore, the phylogenetic analyses revealed a low degree of geographic structure in the mountain tapir.

Temporal splits within the genus Tapirus

With the Bayesian procedure we determined a temporal separation of the ancestor of *T. bairdii* relative to the ancestor of the South American tapirs ranging from 10.5 to 4.63 MYA (95% HPD; μ = 8.1 MYA). The temporal split between the ancestors of *T. terrestris* and *T. pinchaque* oscillated from 7.42 to 3.27 MYA (95% HPD; μ = 3.7 MYA). In our BI tree, the mitochondrial haplotype diversification within *T. terrestris* began somewhere between 5.34 to 2.36 MYA (95% HPD; μ = 3.3 MYA). Within *T. pinchaque*, this process began around 6.09–2.68 MYA (95% HPD), with μ = 2.9 MYA.

Our ML tree with 93 specimens showed reciprocal monophyly between *T. terrestris* and *T. pinchaque*. (Fig. 4) Also, the mitochondrial data support *T. kabomani* as a clade within *T. terrestris*.

Discussion

Genetic diversity, heterogeneity, demographic changes and absence of spatial structure in T. pinchaque

Our nucleotide diversity values for *T. pinchaque* ($\pi = 0.004 \pm 0.0003$) are considerably lower than estimates for *T. terrestris* reported by other studies. Ruiz-García et al. (2015) estimated a value of $\pi = 0.0114 \pm 0.0003$ for a wide geographical sample of *T. terrestris*.

The overall sample of *T. pinchaque* has about three times lower genetic diversity than *T. terrestris* for mitochondrial genes. However, *T. pinchaque* presented a higher nucleotide diversity than *T. bairdii* ($\pi = 0.0025 \pm 0.0005$; Ruiz-García et al., 2012), although the geographical distribution and population size of *T. pinchaque* are considerably smaller than in *T. bairdii*. Cavelier et al. (2010) estimated a maximum population of 5,000 to 5,700 mountain tapirs, based on existing suitable habitat, and estimated densities for this tapir species. However, the same authors considered that the population censuses are really smaller than 5,000 animals, and that the overall mountain tapir population could be around 2,650 to 2,850 individuals.

Currently, seven (Lizcano et al., 2002) or eight (Cavelier et al., 2010) protected areas contained mountain tapirs in Colombia and six protected areas contained mountain tapirs in Ecuador. Our molecular results, therefore, adequately represent the population of *T. pinchaque* within the Colombian Central Cordillera. However, we could not sequence any individual from the Eastern Cordillera nor from the Western Cordillera (in the supposed case that this species lives in that area). It would be highly beneficial to analyze individuals of these Colombian cordilleras because their populations are probably extremely small and at an elevated risk of extinction. However, our results are representative of all the Ecuadorian *T. pinchaque* populations because we analyzed samples from the three main areas where this species lives in Ecuador (Northern Eastern Cordillera: Cayambe-Coca National Park;

Table 6
Genetic heterogeneity and gene flow (Nm) statistics for different *Tapirus pinchaque* sets at 15 mitochondrial genes: (A) Six *T. pinchaque* populations (three in Colombian and three in Ecuador); (B) Population F_{ST} (below) and Nm (above) pairs among the six *T. pinchaque* populations analyzed. *Significant Probability (P < 0.05). inf = infinite. 1 = Los Nevados National Park (Colombia); 2 = Tolima Department (Colombia); 3 = Purace National Park (Colombia); 4 = Cayambe-Coca National Park and other areas in Napo Province (Ecuador); 5 = Sangay National Park (Ecuador); Podocarpus National Park (Ecuador).

(A) Six *T. pinchaque* populations (three in Colombian and three in Ecuador)

Statistics	Gene flow
G_{ST} = 0.0518	Nm = 9.15
γ_{ST} = 0.1612*	Nm = 2.60
N_{ST} = 0.2389*	Nm = 1.59
F_{ST} = 0.2401*	Nm = 1.58

(B) Population F_{ST} (below) and Nm (above) pairs among the six *T. pinchaque* populations analyzed

	1	2	3	4	5	6
1	0.000	16.42	0.34	2.85	0.79	1.35
2	0.029	0.000	76.55	8.80	67.21	inf
3	0.595*	0.006	0.000	1.06	0.64	0.20
4	0.149*	0.054	0.319	0.000	4.75	inf
5	0.387*	0.007	0.438	0.095	0.000	inf
6	0.270	0.000	0.115	0.000	0.000	0.000

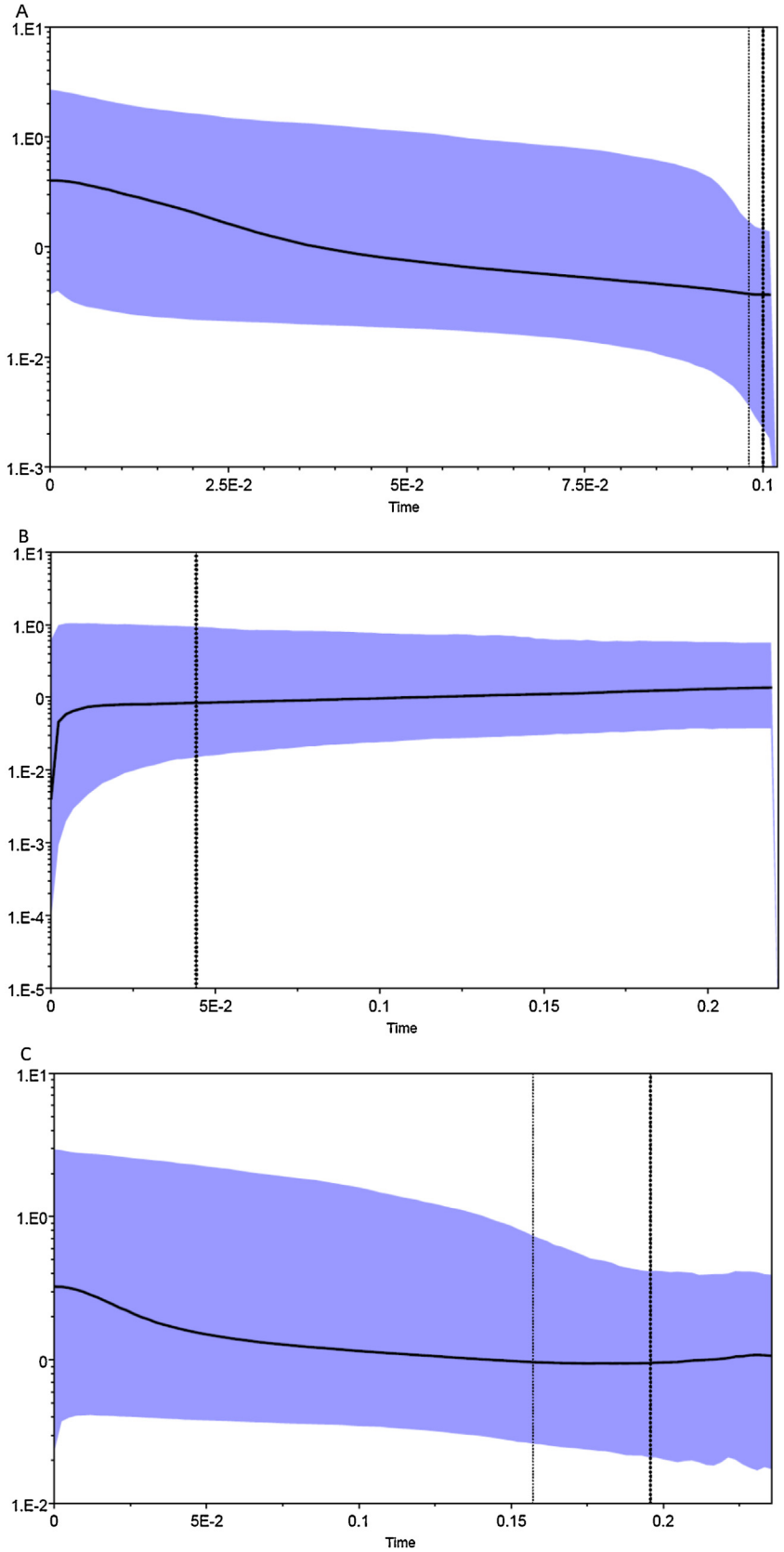

Fig. 2. Bayesian skyline plot (BSP) analyses applied to the *Tapirus pinchaque* population studied at the mitochondrial DNA. (A) Overall *Tapirus pinchaque* sample; (B) Colombian mountain tapir sample; (C) Ecuadorian mountain tapir sample. On the x-axis, time in millions of years; on the y-axis, size of female effective population. Dashed vertical lines are initial points where demographic changes were detected.

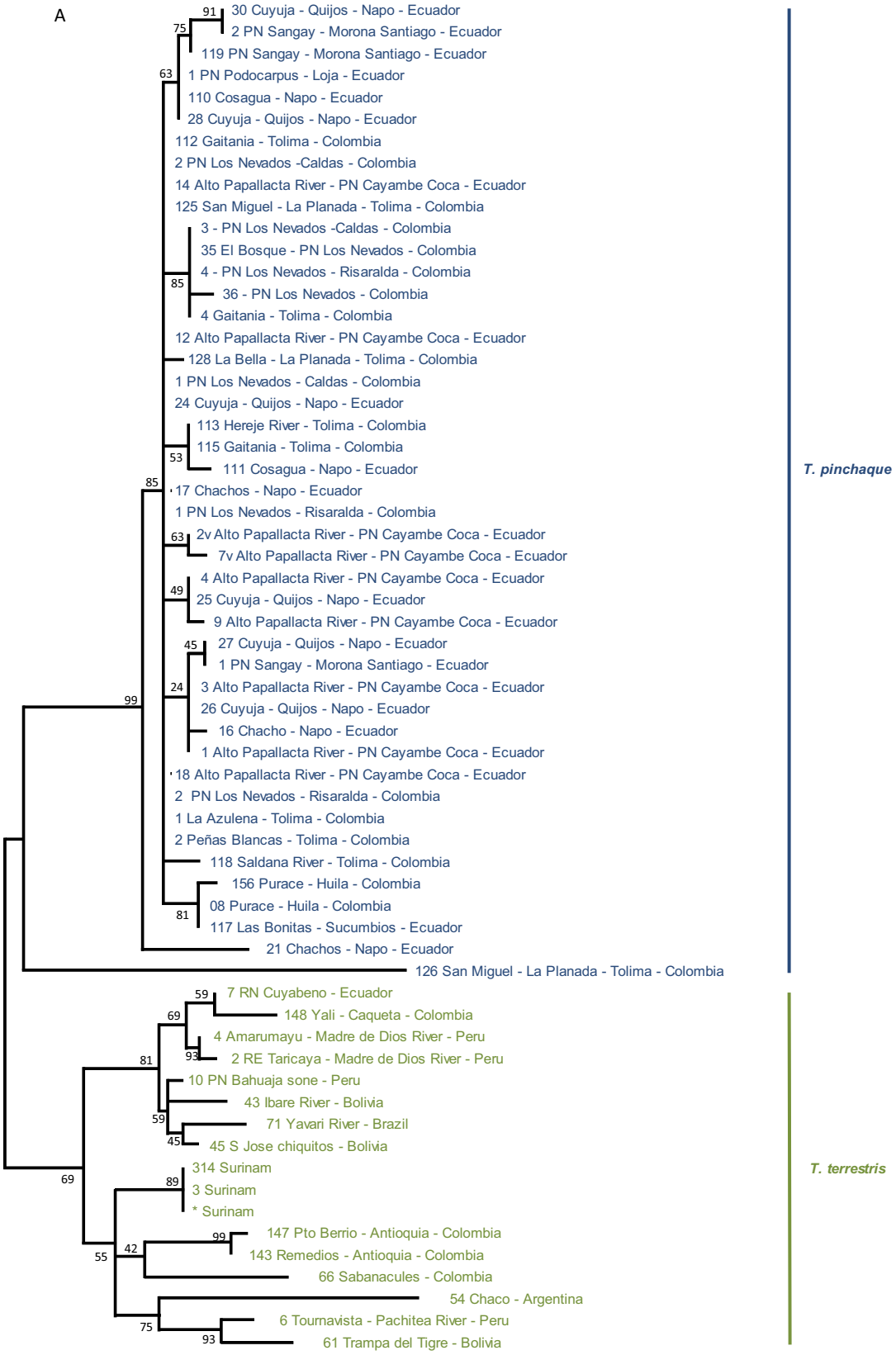

Fig. 3. (A) Maximum likelihood tree with 45 *Tapirus pinchaque*, plus 17 *T. terrestris* by employing sequences of 15 concatenated mitochondrial genes. (B) The same with a Bayesian tree. The number in the nodes are bootstrap percentages higher than 50% for the first tree; the number in the nodes are posterior probabilities higher than 0.5 for the second tree.

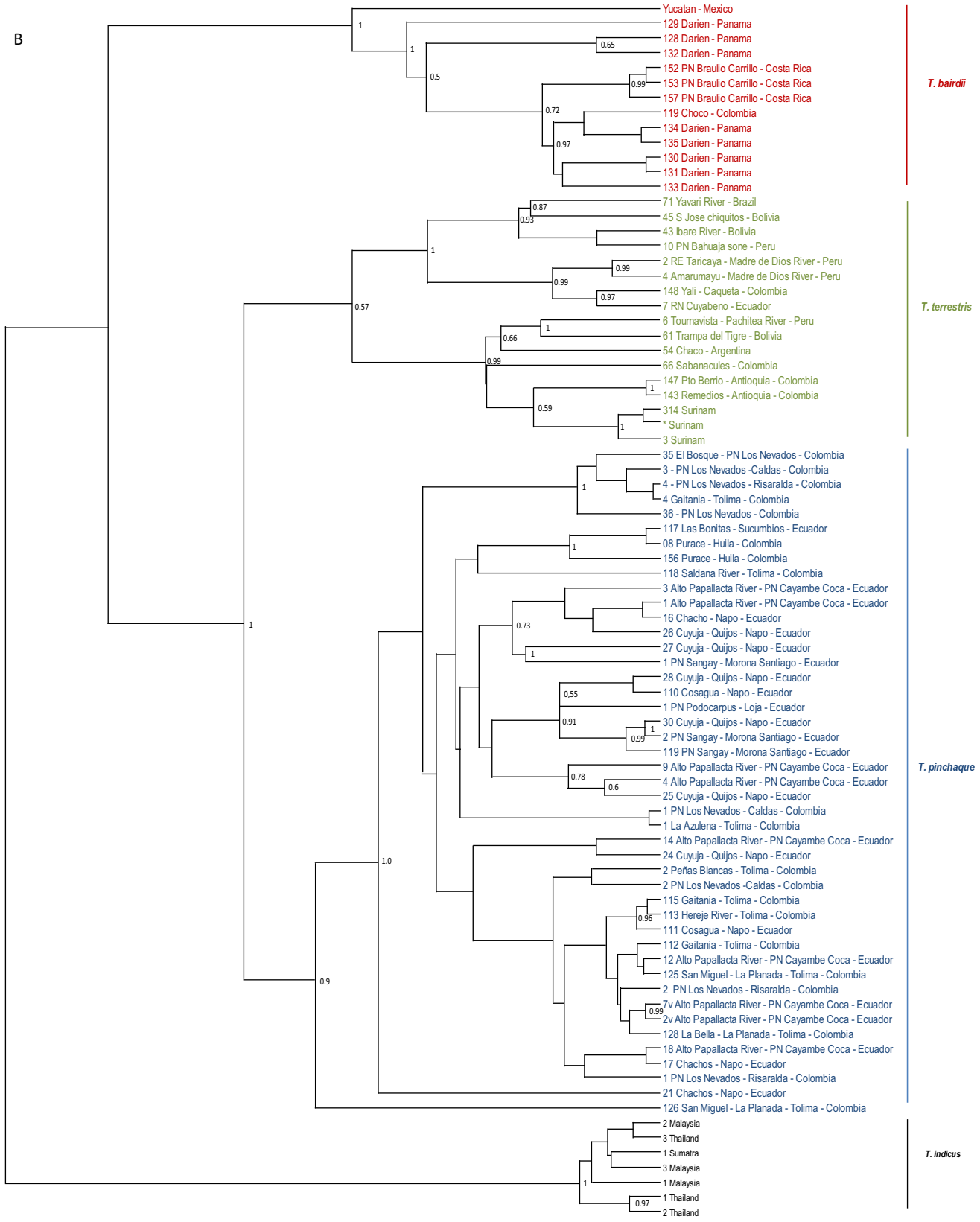

Fig. 3. (*Continued*)

Central Eastern Cordillera: Sangay National Park; and Southern Eastern Cordillera: Podocarpus National Park). Thus, our genetic diversity estimates for the mountain tapir population of Ecuador should be more accurate and realistic than what we estimated for the Colombian population.

Our BSP analysis indicated a strong population decrease in the last 5,000 years for the Colombian population, which coincided with one of the dry periods in the Holocene after the Optimum Climaticum. Following Rothlisberger (1987), around 6,300 YA, there was a significant increase in temperature especially in Southern

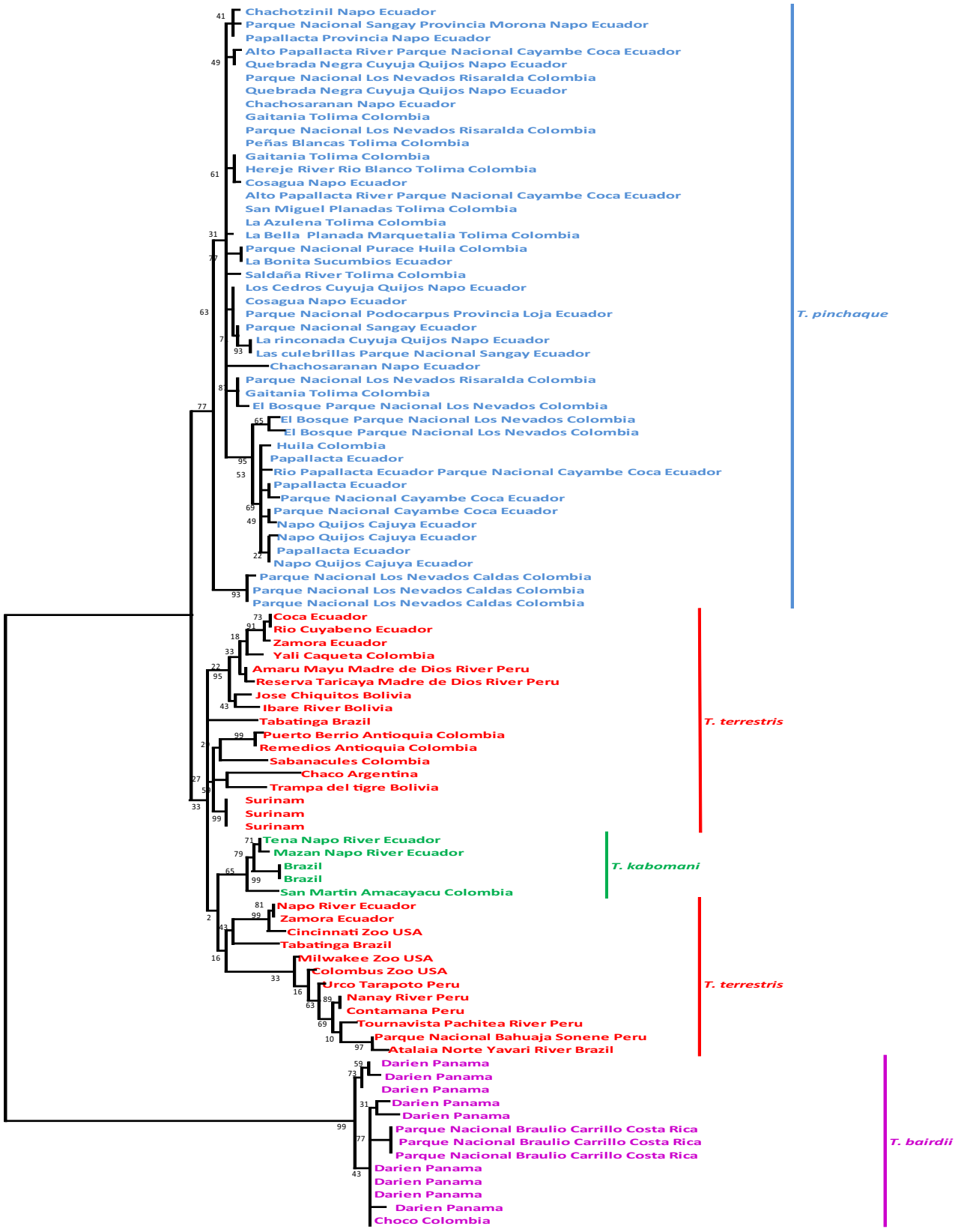

Fig. 4. Maximum likelihood tree for 93 Neotropical tapir specimens (including *T. pinchaque*, *T. terrestris*, *T. bairdii* and *T. kabomani*) for three concatenated mitochondrial genes (*Cyt-b* + *COI* + *COII*).

South America as well as in the Central Andes. This dry period, around 5,000–6,000 YA, was also detected in the Amazon, Caqueta and lower Magdalena River basins as well as in the Andean lagoons of Colombia and Peru (Thompson et al., 1995; Van der Hammen, 2001). These climatic changes together with hunting by humans (Frisson, 1998) might explain the population decline for *T. pinchaque* in the area of Colombia. This fact also coincides with that found by Ruiz-García et al. (2015) for the *T. terrestris* population of

Northern Colombia and Northwestern Venezuela, which showed an extreme population decrease in the last thousand years. In contrast, the Ecuadorian population showed a relatively recent population expansion (around 10,000 YA). The most intense cold and dry period of the last Pleistocene glaciation occurred 26,000 to 14,000 YA (Van der Hammen and Cleff, 1992). This period was related to the maximum extension of the glaciers in the northern hemisphere and the complete disappearance of some lagoons near Bogota (Van Geel and van der Hammen, 1973). Later, from 14,000 YA to 10,000 YA (Tardiglacial), the climate became hotter and wetter. After these dry and cold periods the mountain tapirs in Ecuador probably experienced a population increase due to better climatic conditions. It seems that the climatic Holocene event which affected the mountain tapirs in Colombia did not affect the tapirs in Ecuador. An unequal impact of hunting in these areas could also explain these differences.

Non-significant or very limited genetic heterogeneity among mountain tapir populations means that historical gene flow has been sufficient enough to avoid genetic differentiation, although the Andean habitat is currently extremely fragmented. Fragmentation might be very recent, thus preventing genetic differentiation of the mountain tapir populations. Another possibility might be high gene flow. This has been demonstrated for *T. terrestris* (Pinho et al., 2013) but not yet for *T. pinchaque*. The non-existence of spatial genetic structure is an interesting fact from a conservation perspective because there should not be genetic consequences if animals are translocated.

Phylogenetics of the Neotropical tapirs and evolutionary patterns in T. pinchaque

Hershkovitz (1954), Haffer (1970) and Holanda and Ferrero (2013) used morphological characters and determined that the lineage which gave rise to *T. pinchaque* was the oldest of the current Neotropical tapir species. The conclusion by Hershkovitz (1954) was based on the close similarity of cranial contours to some fossil tapir skull which has a less specialized proboscis than current tapir species.

The adaptation of *T. pinchaque* to the temperate and cold climates motivated Hershkovitz (1954) to affirm that this species appeared in a temperate climate before the completion of the Panamanian land bridge with the minimum age of this species being around 3–4 MYA. Lack of fossil records of *T. pinchaque* makes it difficult to support this hypothesis. Additionally, no molecular results support the fact that the ancestor of *T. pinchaque* is the most ancient of the current Neotropical tapirs. All the molecular studies have shown that *T. pinchaque* and *T. terrestris* are sister groups (Ashley et al., 1996; Norman and Ashley, 2000; Ruiz-García et al., 2012, 2015).

The problem of using craniometrical and teeth data when dealing with recently differentiated organisms is that nobody knows the degree of phenotypic plasticity of these characters and their genetic basis. For instance, Hershkovitz (1954) found a variable trait in the first upper premolar of *T. pinchaque*. Ecuadorian specimens show the simple condition, with the cinguloid shelf absent, whereas the first Colombian skull analyzed of *T. pinchaque* had a premolar like another *Tapirus* species. For this reason, two subspecies (*T. p. pinchaque* and *T. p. leucogenys*) were identified within the mountain tapir. In contrast, our mitogenomic analysis shows that haplotypes from animals from both distributions are intermixed and it could represent a unique taxon and/or they diverged very recently. Molecular data do not support the paleontological hypothesis of Holanda and Ferrero (2013) nor the hypothesis of Hershkovitz (1954) that *T. pinchaque* and *T. terrestris* correspond to different lineages of tapirs. Our mitogenomic results are more in line with the paleontological view of Hulbert and Wallace (2005) and Hulbert (2010) because they consider that *T. pinchaque* and *T. terrestris* form a monophyletic group.

Another question is that the genetic distances between *T. pinchaque* and *T. terrestris* are very small for traditional fully differentiated species. Kartavtsev (2011) analyzed sequences of *COI* from 20,731 vertebrate and invertebrate animal species and estimated the average distance data for five different groups. He calculated $0.89\% \pm 0.16\%$ for populations within species and $3.78\% \pm 1.18\%$ for subspecies or semispecies. For species within a genus he calculated $11.06\% \pm 0.53\%$. For this gene, the genetic differentiation between *T. terrestris* and *T. pinchaque* was only 1%, a value for populations or subspecies within species. However, we consider *T. pinchaque* a full species by different criteria. Using the PSC, it formed a reciprocally monophyletic clade with *T. terrestris* (including *T. kabomani*) and using the BSC, it is a reproductive unit because no natural nor captivity hybridization with *T. terrestris* has been reported due probably to important differences in their karyotypes, although its molecular differentiation is minor. Barongi (1993) and Houck et al. (2000) showed 2N = 80 and FN = 80–92 for *T. terrestris*, but 2N = 76 and FN = 80–84 for *T. pinchaque*. The correlation of important chromosome differences but with very low mitochondrial divergence is typical of peripatric or parapatric chromosomal speciation (Lewis, 1966; White, 1968, 1978; King, 1993). This has been reported in other Peryssodactyla, such as in the different zebra species (Bush, 1981). Additionally, ecological isolation could be another cause of speciation for *T. pinchaque* (Ecological Species Concept).

But when did the last common ancestor of *T. terrestris* and *T. pinchaque* live? Although, Cozzuol et al. (2013) (see Fig. 7 of these authors) claimed that this could have happened around 0.1–0.3 MYA, we believe it occurred considerably before (1.5–7 MYA) for two reasons: (1) Ashley et al. (1996) and Norman and Ashley (2000) estimated a split around 1.5–3 MYA with two mitochondrial genes. Ruiz-García et al. (2012) showed a temporal split between the ancestors of both tapir species of around 3.8 MYA for a Bayesian tree with some temporal priors. With other procedures, without priors, these authors estimated a split of around 1.6 MYA. Our present results with mitogenomics showed a range of 7.4–3.3 MYA with a mean around 3.7 MYA. (2) Downer (2001) showed that a significant portion of the original high Andean flora from Northern Peru and further north could have depended upon the mountain tapir as a seed disperser. This Flora appeared during the geologically recent Andean rise that began after the Quaternary Period (late Cenozoic) around 2.5 million MYA (Simpson, 1979; Benton, 1991). This author showed indirect ecological evidence in favor of the presence of the mountain tapir in the last 2.5 MYA within the Northern Andean Cordilleras.

Molecular evidence against T. kabomani *as a new species*

Our results showed reciprocal monophyly between *T. pinchaque* and *T. terrestris* and that *T. kabomani* was a clade nested within *T. terrestris*. Thus, our mitochondrial analyses did not validate *T. kabomani* as a new species as suggested by Cozzuol et al. (2013). Our results agree with those of Voss et al. (2014), who claimed that *T. kabomani* is not a different species from *T. terrestris*. Cozzuol et al. (2013) analyzed a very small sample of *T. pinchaque* that probably did not represent the total mitochondrial genetic diversity of this species. By chance, it was nested within the genetic diversity of *T. terrestris* because of the small genetic distances between both taxa.

Additionally, we did not detect a correlation between the morphotype proposed by Cozzuol et al. (2013) for *T. kabomani* and the *T. kabomani* mitochondrial haplotypes. Cozzuol et al. (2013) described *T. kabomani* with a lower sagittal crest, a smaller size and a darker color than the typical morphotype of *T. terrestris*. However, the Colombian (Amacayacu River) and the Ecuadorian (upper Napo River) animals which showed haplotypes of the *T. kabomani*

presented typical morphotypes of *T. terrestris* (we couldn't determine the morphotype of the Peruvian specimen because the sample came from a piece of skin of a hunted animal). Contrarily, we have found and sampled small-sized and dark lowland tapirs similar to the morphotype described by Cozzuol et al. (2013) for *T. kabomani* but they presented haplotypes of other *T. terrestris* haplogroups.

We agree with Zachos (2015) who stated that species are such fundamental units that they should not be introduced carelessly and that species description and splitting based on simple morphometric differences (even significant ones) or phylogenetic relationships derived from limited molecular datasets (for instance, only mtDNA) should be strongly discouraged. They may serve to support conclusions derived from larger and more complete datasets, but are not enough on their own. Therefore, additional data are needed to test if *T. kabomani* is a new species. Nuclear DNA should be analyzed to confirm the findings revealed by mitogenomics for *T. pinchaque*.

Acknowledgements

The authors are grateful for help and permissions from the Ecuadorian Ministerio del Ambiente, Ecofondo, Ecociencia, Zoological Foundation of Ecuador, Instituto para la Conservación y Capacitación Ambiental (ICCA), Tapir Specialist Groups-from Colombia and Ecuador, Andean Bear Foundation in USA and Ecuador and to the Von Humboldt Institute in Villa de Leyva (Colombia). Thanks are also given to Carlos Urgiles, Freddy Gallo, Leopoldo Gómez, Fabian Ascanta and Olimpo Gómez to help to obtain samples of *T. pinchaque* in Cuyuja and in Oyacachi (Ecuador). Also thanks to Dr. Diana Alvarez, Pablo Escobar-Armel and Luisa Fernanda Castellanos-Mora for their respective help in obtaining *T. pinchaque* and *T. terrestris* samples during the last 18 years in Colombia.

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Autom. Control AC-19, 716–723.
Ashley, M.V., Norman, J.E., Stross, L., 1996. Phylogenetic analysis of the perissodactylan family Tapiridae using mitochondrial cytochrome c oxidase (COII) sequences. J. Mammal. Evol. 3, 315–326.
Barongi, R.A., 1993. Husbandry and conservation of tapirs *Tapirus* spp. Int. Zoo Yb. 32, 7–15.
Bensasson, D., Zhang, D.-X., Hartl, D.L., Hewitt, G.M., 2001. Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends Ecol. Evol. 16, 314–321.
Benton, M., 1991. The Rise of Mammals. Crescent Books, New York.
Bohonak, A.J., 2002. IBD (Isolation by Distance): a program for analyses of isolation by distance. J. Hered. 93, 153–154.
Brooks, D.M., Bodmer, R.E., Matola, S., 1997. Tapir-Status Survey and Conservation Action Plan. IUCN/SSC Tapir Specialist Group, IUCN Gland & Cambridge.
Bush, G.L., 1981. Stasipatric speciation and rapid evolution in animals. In: Atchley, W.R., Woodruff, D.S. (Eds.), Evolution and Speciation. Essays in Honor of M. J. D. White. Cambridge University Press, Cambridge, USA, pp. 201–218.
Cavelier, J., Lizcano, D., Yerena, E., Downer, C., 2010. The mountain tapir (*Tapirus pinchaque*) and Andean bear (*Tremarctos ornatus*): two charismatic, large mammals in South American tropical montane cloud forests. In: Bouijnacel, L.A., Scabena, F.N., Hamilton, L.S. (Eds.), Tropical Montane Cloud Forests: Science for Conservation and Management. Cambridge University Press, Cambridge, USA, pp. 172–181.
Cozzuol, M.A., Clozato, C.L., Holanda, E.C., Rodrigues, F.H.G., Nienow, S., Thoisy, B., Redondo, R.A.F., Santos, F.R., 2013. A new species of tapir from the Amazon. J. Mammal. 94, 1331–1345.
Downer, C.C., 2001. Observations on the diet and habitat of the mountain tapir (*Tapirus pinchaque*). J. Zool. 254, 279–291.
Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.
Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLOS Biol. 4, e88.
Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.
Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.
Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.
Ferrero, B.S., Noriega, J.I., 2007. A new upper Pleistocene tapir from Argentina: remarks on the phylogenetics and diversification of Neotropical Tapiridae. J. Vert. Paleontol. 27, 504–511.
Frisson, G.C., 1998. Paleoindian large mammal hunters on the plains of North America. Proc. Natl. Acad. Sci. U.S.A. 95, 14,576–14,583.
Haffer, J., 1970. Geologic-climatic history and zoogeographic significance of the Uraba region in northwestern Colombia. Caldasia 10, 603–636.
Hershkovitz, P., 1954. Mammals of Northern Colombia. Preliminary Report no. 7. Tapirs (genus *Tapirus*), with a systematic review of American species. Proc. U.S. Nat. Mus. 103, 465–496.
Hershkovitz, P., 1966. Mice, land bridges and Latin American faunal interchange. In: Wenzel, R.L., Tipton, V.J. (Eds.), Ectoparasites of Panama. Field Museum of Natural History, Chicago, pp. 725–751.
Holanda, E.C., Ferigolo, J., Ribeiro, A.M., 2011. New *Tapirus* species (Mammalia: Perissodactyla: Tapiridae) from the upper Pleistocene of Amazonia, Brazil. J. Mammal. 92, 111–120.
Holanda, E.C., Ferrero, B.C., 2013. Reappraisal of the genus *Tapirus* (Perissodactyla, Tapiridae): systematics and phylogenetic affinities of the South American tapirs. J. Mammal. Evol. 20, 33–44.
Houck, M.L., Kingswood, S.C., Kumamoto, A.T., 2000. Comparative cytogenetics of tapirs, genus *Tapirus* (Perissodactyla, Tapiridae). Cytogenet. Cell Genet. 89, 110–115.
Hudson, R.R., Boss, D.D., Kaplan, N.L., 1992. A statistical test for detecting population subdivision. Mol. Biol. Evol. 9, 138–151.
Hulbert, R.C., 2010. A new early Pleistocene tapir (Mammalia: Perissodactyla) from Florida, with a review of Blancan tapirs from the state. Bull. Florida Museum Nat. Hist. 49, 67–126.
Hulbert, R.C., Wallace, S.C., 2005. Phylogenetic analysis of late Cenozoic *Tapirus* (Mammalia, Perissodactyla). J. Vert. Paleontol. 25 (Suppl. 3), 72A.
Kartavtsev, Y., 2011. Divergence at *Cyt-b* and *Co-1* mtDNA genes on different taxonomic levels and genetics of speciation in animals. Mitochondrial DNA 22, 55–65.
Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
King, M., 1993. Species Evolution. Cambridge University Press, Cambridge.
Lewis, H., 1966. Speciation in flowering plants. Science 152, 167–172.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452, http://dx.doi.org/10.1093/bioinformatics/btp187.
Lizcano, D.J.V., Pizarro, J., Cavelier, J., Carmona, J., 2002. Geographic distribution and population size of the mountain tapir (*Tapirus pinchaque*) in Colombia. J. Biogeogr. 28, 1–9.
Lizcano, D.J., Guarnizo, A., Suárez, J., Flores, F.K., Montenegro, O., 2006. Danta de páramo *Tapirus pinchaque*. In: Rodríguez-M, J.V., Alberico, M., Trujillo, F., Jorgenson, J. (Eds.), Libro rojo de los mamíferos de Colombia. Serie libros rojos de especies amenazadas de Colombia. Conservación Internacional Colombia, Ministerio de Ambiente, Vivienda y Desarrollo Territorial, Bogotá, Colombia, 173 pp.
Mantel, N.A., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.
Mau, B., 1996. Bayesian Phylogenetic Inference via Markov Chain Monte Carlo Methods. University of Wisconsin, Madison.
Mau, B., Newton, M., Larget, B., 1999. Bayesian phylogenetic inference via Markov chain Monte Carlo methods. Biometrics 55, 1–12.
Norman, J., Ashley, M., 2000. Phylogenetics of Perissodactyla and tests of the molecular clock. J. Mol. Evol. 50, 11–21.
Pinho, G.M., Goncalves da Silva, A., Hrbeck, T., Farias, I.P., 2013. Comportamento Social de antas (*Tapirus terrestris*): Relacoes de parentesco em uma paisagem fragmentada, Amazonia Central, Brasil. In: I Congreso Latinoamericano de tapires, 8–11 May 2013, Puyo-Pastaza, Ecuador.
Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.
Rannala, B., Yang, Z., 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. 43, 304–311.
Rothlisberger, F., 1987. 10,000 jahre gletschergeschichte der erde. Verlag Sauerlander, Aarau, Switzerland, pp. 1–225.
Ruiz-García, M., Vásquez, C., Pinedo-Castro, M., Sandoval, S., Kaston, F., Thoisy, B., Shostell, J.M., 2012. Phylogeography of the mountain tapir (*Tapirus pinchaque*) and the Central American tapir (*Tapirus bairdii*) and the molecular origins of the three South-American tapirs. In: Anamthawat-Jónsson, K. (Ed.), Current Topics in Phylogenetics and Phylogeography of Terrestrial and Aquatic Systems. InTech, Rijeka, Croatia, pp. 83–116.
Ruiz-García, M., Vásquez, C., Sandoval, S., Kaston, F., Luengas-Villamil, K., Shostell, J.M., 2015. Phylogeography and spatial structure of the lowland tapir (*Tapirus terrestris*, Perissodactyla: Tapiridae) in South America. Mitochondrial DNA 26, 1–9, http://dx.doi.org/10.3109/19401736.2015.1022766 (online).
Schauenberg, P., 1969. Contribution a letude du Tapir pinchaque, *Tapirus pinchaque* Roulin 1829. Rev. Suisse Zool. 76, 211–256.
Simpson, B.B., 1979. Quaternary biogeography of the high montane regions of South America. In: Duellman, W.E. (Ed.), The South American Herpetofauna: Its Origin, Evolution and Dispersal. Monograph of the Museum of Natural History No. 7. University of Kansas, Lawrence, pp. 157–188.

Sokal, R.R., Rohlf, F.J., 1981. Biometry: The Principles and Practice of Statistics in Biological Research, 2nd ed. Freeman, San Francisco.
Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony and other methods., pp. 1–142 http://paup.csit.fsu.edu.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Thalmann, O., Hebler, J., Poinar, H.N., Paabo, S., Vigilant, L., 2004. Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other apes. Mol. Ecol. 13, 321–335.
Thoisy, B., Goncalves da Silva, A., Ruiz-García, M., Tapia, A., Ramirez, O., Arana, M., Quse, V., Paz-y-Miño, C., Tobler, M., Pedraza, C., Lavergne, A., 2010. Population history, phylogeography, and conservation genetics of the last Neotropical mega-herbivore, the Lowland tapir (*Tapirus terrestris*). BMC Evol. Biol. 10, 278–295.
Thompson, L.G., Mosley, E., Davies, M.E., Lin, P.N., Henderson, K.A., Coledal, J., Bolzan, J.F., Liu, K.B., 1995. Huascarán, Perú. Science 269, 46–50.
Tirira, D., 2011. Libro Rojo de los Mamíferos del Ecuador. Publicación especial de los Mamíferos del Ecuador No. 8. Pontificia Universidad Católica del Ecuador and Ministerio de Medio Ambiente, Quito, Ecuador.
Vaidya, G., Lohman, D.J., Meier, R., 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27, 171–180.
Van der Hammen, T., 2001. Paleoecology of Amazonia. In: Guimaraes Vieira, I.C., Silva, J.M.C., Oren, D.C., D'Incao, M.A.D. (Eds.), Diversidade biológica e cultural da Amazonia. Museu Paraense Emilio Goeldi, Belem, Brazil, pp. 19–44.
Van der Hammen, T., Cleff, A.M., 1992. Holocene changes of rainfall and river discharge in northern South America and the El Niño phenomenon. Erdkunde 46, 252–256.
Van Geel, B., van der Hammen, T., 1973. Upper Quaternary vegetational and climatic sequence of the Fúquene area (Eastern Cordillera, Colombia). Palaeogeogr. Palaeoclimatol. Palaeoecol. 14, 9–92.
Voss, R.S., Helgen, K.M., Jansa, S.A., 2014. Extraordinary claims require extraordinary evidence: a comment on Cozzuol et al. (2013). J. Mammal. 95, 893–898.
White, M.J.D., 1968. Models of speciation. New concepts suggest that the classical sympatric and allopatric models are not the only alternatives. Science 159, 1065–1070.
White, M.J.D., 1978. Modes of Speciation. W.H. Freeman, San Francisco.
Wright, S., 1951. The genetical structure of populations. Ann. Eugen. 15, 323–354.
Zachos, F.E., 2015. Tree thinking and species delimitation: guidelines for taxonomy and phylogenetic terminology. Mammal. Biol., http://dx.doi.org/10.1016/j.mambio.2015.10.002 (in press; available online 22 October 2015).